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Molecular Dynamics Simulations of Ligand Recognition upon Binding Antithrombin: A MM/GBSA Approach

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Abstract. A high throughput virtual screening pipeline has been extended from single energetically minimized structure Molecular Mechanics/Generalized Born Surface Area (MM/GBSA) rescoring to ensemble-average MM/GBSA rescoring. For validation, the binding affinities of a series of antithrombin ligands have been calculated by using the two MM/GBSA rescoring methods. The correlation coefficient (R^2) of calculated and experimental binding free energies has been improved from 0.36 (for single-structure MM/GBSA rescoring) to 0.69 (for ensemble-average one). Decomposition of the calculated binding free energy reveals the electrostatic interactions in both solute and solvent play an important role in determining the binding free energy. The increasing negative charge of the compounds provides a more favorable electrostatic energy change but creates a higher penalty for the solvation free energy. Such a penalty is compensated by the electrostatic energy change, which results in better binding affinity. The best binder has the highest ligand efficiency.

Keywords: MM/GBSA, Molecular dynamics, Binding Affinity, Antithrombin, Heparin

1 Introduction

High throughput virtual screening is an important tool for the computer-aided drug discovery. We have developed a high throughput virtual screening pipeline for *in-silico* screening of virtual compound database using high performance computing (HPC) [1]. The previous pipeline consists of four modules: receptor/target preparation, ligand preparation, VinaLC docking calculation [2], and single-structure MM/GBSA rescoring. All modules are paralleled to exploit typical cluster-type supercomputers. The MM/GBSA method is selected for rescoring because it is the fastest force-field based method that computes the free energy of binding, as compared to the other computational free energy methods, such as free energy perturbation (FEP) and thermodynamic integration (TI) methods [3]. The MM/GBSA method has been widely exploited in free energy calculations [4, 5]. One of the notable features of this

pipeline is an automated receptor preparation scheme with unsupervised binding site identification, which enables automatically running of whole pipeline without too much human intervention. A similar approach has been developed by Professor Horacio Perez-Sanchez and co-workers to improve drug discovery using massively parallel GPU hardware instead of supercomputers [6]. Their GPU-based program, BINDSURF [7], takes advantage of massively parallel and high arithmetic intensity of GUPs to speed-up the calculation in low cost desktop machine.

In this study, we have extended our pipeline from single-structure to ensemble-average MM/GSBA rescoring. To validate the new approach, we have gathered a panel of antithrombin ligands (Figure 1), including heparin and non-polysaccharide scaffold compounds. For the purpose of comparison, both single-structure and ensemble-average MM/GSBA rescoring are employed in the binding affinity calculations of antithrombin ligands. We must point out that estimation/calculations of entropy term are tricky. In most scenarios, the entropy term is neglected in the calculation for relative free binding energies. Quite a few researchers dispute the benefits of including the entropy term, which can be a major source of error due to the drawback of the entropy calculation method [8, 9], despite others who advocate its usage [10]. We choose to neglect the entropy term in our calculations.

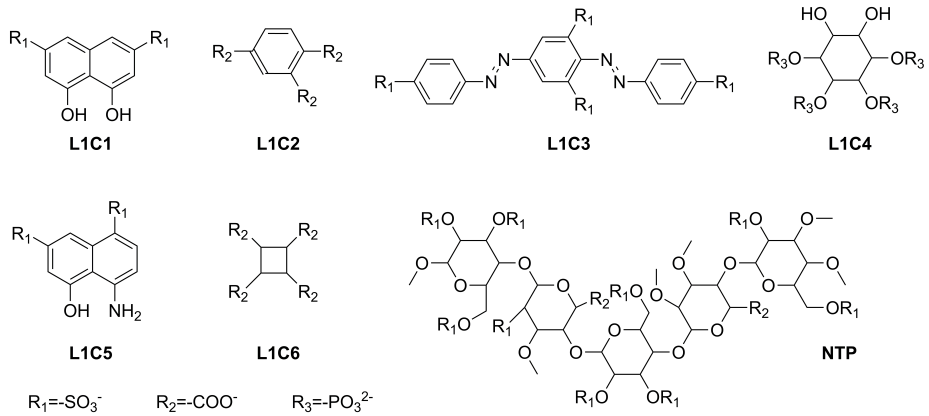

Fig. 1. Compounds targeting antithrombin. Compound NTP is a synthetic pentasaccharide compound from the crystal structure (PDB ID: 1AZX).

Antithrombin is a glycoprotein that plays a crucial role in the regulation of blood coagulation by inactivating several enzymes of the coagulation system and, thus, is an important drug target for the anticoagulant treatment. Antithrombin has two major isoforms, α and β, in the blood circulation [11]. α-Antithrombin is the dominant form of antithrombin and consists of 432 amino acids with 4 glycosylation sites, where an oligosaccharide occupying each glycosylation sites [12]. Heparin is the first compound that was identified and used as an anticoagulant and antithrombotic agent. It is a sulfated polysaccharide containing a specific pentasaccharide fragment (Figure 1, NTP) that binds and activates the antithrombin [13]. This binding localized the function of antithrombin to inhibition of serine proteases in the coagulation cascade in the

bloodstream, which allows coagulant activity in damaged tissue outside the vascular system [12].

Due to increasing interests in clinical applications, computational studies have been carried out to investigate the structure and behavior of antithrombin. Verli and co-workers performed molecular dynamics simulations to study the induced-fit mechanism of the antithrombin-heparin interaction and effects of glycosylation on heparin binding [14, 15]. Several detailed conformational changes associated with heparin binding to antithrombin were revealed. They also confirmed an intermediate state between the native and activated forms of antithrombin. Because of the weak surface complementarity and the high charge density of the sulfated sugar chain, the docking of heparin to its protein partners presents a challenging task for computational docking. Wade and Bitomsky developed a protocol that can predict the heparin binding site correctly [16]. Navarro-Fernandez and colleagues screened a large database *in silico* and identified a new, non-polysaccharide scaffold able to interact with the heparin binding domain of antithrombin [17]. They predicted D-myo-inositol 3,4,5,6-tetrakisphosphate (Figure1, L1C4) to strongly interact with antithrombin, which was confirmed by experimental binding affinity study.

2 Method

The MM/GBSA calculations are applied to the antithrombin (PDB ID: 1AZX) and its seven ligands (Figure 1) by using our in-house developed pipeline [1, 2] and Amber molecular simulation package [18]. The Amber forcefield f99SB [18] is employed in the calculation for the antithrombin receptor. Ligands use the Amber GAFF forcefield [19] as determined by the Antechamber program [20] in the Amber package. Partial charges of ligands are calculated using the AM1-BCC method [21]. The fourth module of the pipeline is employed for the single-structure MM/GBSA calculation, where the receptor-ligand complexes are energetically minimized by the MM/GBSA method implemented in the Sander program of the Amber package [22]. The atomic radii developed by Onufriev and coworkers (Amber input parameter igb=5) are chosen for all GB calculations [23]. For the ensemble-average MM/GBSA rescoring, energetically minimized structures from single-structure MM/GBSA rescoring are served as initial structures. The systems are heated from 0 K to room temperature, 300 K. The MD simulations with a time step of 2 fs for the integration of the equations of motion are carried out at room temperature. The systems are equilibrated at room temperature for 500 ps. Each MD trajectory is followed to 100 ns after equilibrium. Binding affinities of antithrombin and its 7 ligands are calculated by post-processing the ensembles of structures extracted from MD trajectories using MM/GBSA calculations. In the MM/GBSA calculation, the binding free energy between a receptor and a ligand is calculated using the following equations:

$$\Delta G_{\text{bind}} = G_{\text{complex}} - G_{\text{receptor}} - G_{\text{ligand}} \quad (1)$$

$$\Delta G_{\text{bind}} = \Delta H - T\Delta S \approx \Delta E_{\text{gas}} + \Delta G_{\text{sol}} - T\Delta S \quad (2)$$

$$\Delta E_{\text{gas}} = \Delta E_{\text{int}} + \Delta E_{\text{ELE}} + \Delta E_{\text{VDW}} \quad (3)$$

$$\Delta G_{sol} = \Delta G_{GB} + \Delta G_{Surf} \quad (4)$$

The binding free energy (ΔG_{bind}) is decomposed into different energy terms. Because the structures of complex, receptor, and ligand are extracted from the same trajectory, the internal energy change (ΔE_{int}) is canceled. Thus, the gas-phase interaction energy (ΔE_{gas}) between the receptor and the ligand is the sum of electrostatic (ΔE_{ELE}) and van der Waals (ΔE_{VDW}) interaction energies. The solvation free energy (ΔG_{sol}) is divided into the polar and non-polar energy terms. The polar solvation energy (ΔG_{GB}) is calculated by using GB model. The non-polar contribution is calculated based on the solvent-accessible surface area (ΔG_{Surf}). A value of 80 is used for the solvent dielectric constant and the solute dielectric constant is set to 1. The calculated binding free energy (ΔG_{bind}) is the sum of the gas-phase interaction energy and solvation free energy because we neglect the entropy term. The experimental binding free energy is estimated from the experimental dissociation constant (Kd) by the equation:

$$\Delta G_{Exp} = RT \cdot \ln(Kd) \quad (5)$$

where R is the gas constant, and T is the temperature.

3 Results and Discussion

The calculated binding free energies of seven antithrombin ligands using the ensemble-average MM/GBSA rescoring are shown in Table 1 together with their corresponding experimental values. Each calculated binding free energy is averaged from snapshots extracted from 100 ns MD trajectory. Except for Compound L1C1, all the antithrombin ligands have experimental binding free energies. As determined experimentally, Compound L1C4 is the best binder with a Kd value of 0.088 uM [17]. As predicted by the MM/GBSA method, Compound L1C4 has the most negative binding free energy (-308.01 kcal/mol), which is in agreement with the experimental results. The second best binder as predicted by the MM/GBSA calculation is Compound NTP with a calculated binding free energy of -279.57 kcal/mol, confirming the experimental ranking relative to Compound L1C4. Compound L1C2 is predicted to have the worst binding free energy of the six ligands, which is also in agreement with its experimental ranking value. In summary, the MM/GBSA calculations rank the binding affinities of all six antithrombin ligands in same exact order as that of experimental binding free energy rankings.

The calculated binding free energies of six antithrombin ligands using the ensemble-average MM/GBSA rescoring have been plotted against the free energies derived from experimental dissociation constants. The correlation coefficient (R2) is 0.69, which indicates good correlation between the calculated and experimental values. As comparison, the correlation coefficient calculated by single-structure MM/GBSA is only 0.36. Compound NTP was predicted to be the best binder instead of Compound L1C4. Thus, using ensemble-average MM/GBSA rescoring method has significantly

improved the accuracy of the prediction over the single-structure MM/GBSA rescoring.

Table 1. Calculated and experimental binding free energies (kcal/mol) of antithrombin ligands.

Cmpd	ΔE_{ELE}	ΔE_{VDW}	ΔE_{gas}	ΔG_{Surf}	ΔG_{GB}	$\Delta G_{GB\text{-}ELE}$	ΔG_{Sol}	ΔG_{bind}	Kd(uM)	ΔG_{Exp}
L1C1	-552.67	-23.68	-576.35	-2.75	480.12	-72.55	477.37	-98.97	-	-
L1C2	-442.99	0.47	-442.52	-1.20	417.60	-25.39	416.41	-26.11	13700	-2.54
L1C3	-836.77	-39.96	-876.73	-4.06	781.94	-54.83	777.88	-98.85	10.02	-6.81
L1C4	-1599.09	33.02	-1566.07	-2.98	1261.05	-338.04	1258.07	-308.01	0.088	-9.62
L1C5	-613.30	-19.00	-632.31	-2.57	525.82	-87.48	523.25	-109.06	0.69	-8.40
L1C6	-818.73	8.21	-810.52	-1.54	752.94	-65.79	751.41	-59.11	17.52	-6.48
NTP	-2598.87	-60.89	-2659.76	-7.58	2387.77	-211.09	2380.20	-279.57	0.104	-9.52

As shown in Figure 1, all antithrombin ligands contain negatively charged groups, suggesting electrostatic interactions should be a key factor in the binding affinity. Compound NTP has a total charge of -11 and Compound L1C4 has a total charge of -8. By decomposition of the binding free energy, Compound NTP and L1C4 have the largest electrostatic energy changes upon binding in both gas phase (ΔE_{ELE}) and GB solvent ($\Delta G_{GB\text{-}ELE}$). The energy change upon binding in gas phase is equivalent to the energy change upon binding for the solute. Thus, in other words, Compound NTP and L1C4 have the largest electrostatic energy changes upon binding in solute and solvent. In contrast, Compound L1C2 has the smallest electrostatic energy changes in solute and solvent. Although Compound L1C4 has the least favorable of van der Waals energy change upon binding, the electrostatic energy change compensates significantly. For all ligands, the van der Waals energy changes (ΔE_{VDW}) upon binding are less than the electrostatic energy changes (ΔE_{ELE}) by 1-2 orders of magnitude. The contribution of the van der Waals energy change has been overpowered by the electrostatic energy change. Non-polar contribution of solvation free energy of the Compound NTP and L1C3 are more negative than that of the other compounds because the sizes of Compound NTP and L1C3 are larger than the other compounds. Nevertheless, non-polar contributions for all compounds are small. The non-polar contribution is overwhelmed by the polar contribution of solvation free energy. Thus, the two major factors to determine the binding affinity are the electrostatic energy change and solvation free energy change. The larger the total charge of the compound, the larger the penalty cost is for solvation free energy. However, high penalty for large total charge of compound has been paid by the large favorable electrostatic energy chang-

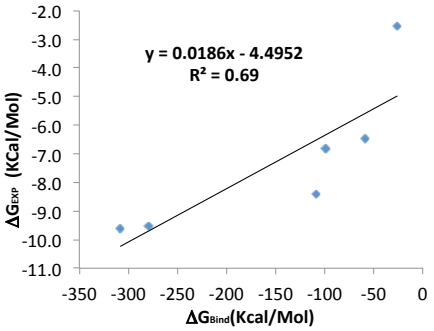

Fig. 2. The scatter plot of calculated MM/GBSA binding free energy versus experimental binding affinity estimated from dissociation constant

es. Although the electrostatic energy change of Compound L1C4 is less than that of Compound NTP, Compound L1C4 needs less compensation for the solvation free energy. Thus, Compound L1C4 is a better binder than Compound NTP.

Hydrogen bonding analysis determines the numbers of hydrogen bonds to antithrombin that are persistent at >20% of the time. For Compound NTP 40 hydrogen bonds are to antithrombin while L1C4 has 25 hydrogen bonds. For Compounds L1C1, L1C2, L1C3, L1C5, and L1C6, that number of hydrogen bonds are 5, 10, 12, 12, and 12 respectively. Taking the molecular weight into account and using a similar approach as Reynolds' ligand efficiency method [24], Compound L1C4 has the highest ligand efficiency.

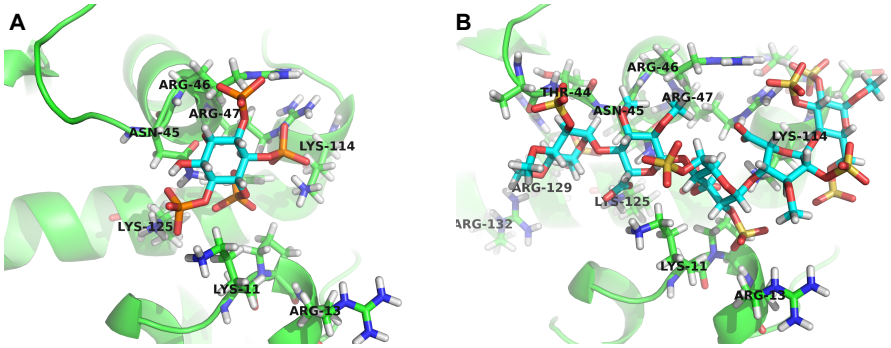

Fig. 3. Initial structures of Compounds L1C4 (A) and NTP (B) complexed with antithrombin.

Compound L1C4 forms double hydrogen bonds with Arg47 (Figure 3A). One hydrogen bond (O6-HH21-NH2) has 94.81% persistence, and the other one (O6-HE-NE) has 89.55%. The average hydrogen bond distances between the heavy atoms are 2.74 Å and 2.70 Å, respectively. Compound L1C4 has strong hydrogen bonds with Arg47, and one of the four phosphate groups from Compound L1C4 is locked to the Arg47. According to the hydrogen bonding analysis, Compound L1C4 is also hydrogen bonded to Arg46, Arg13, Lys114, Lys11, Lys125, and Asn45, which are key residues to the binding process. We found that the binding of Compound L1C4 to antithrombin is non-specific. Except for the phosphate group locked to Arg47, the other three phosphate groups of Compound L1C4 can rotate so that key residues can form hydrogen bonds to different oxygen atoms of phosphate at different times during the MD trajectory. Notably, Arg13 starts far away from Compound L1C4 in the initial conformation. After 8 ns of MD simulation, Arg13 begins to make hydrogen bonds with the phosphate group of Compound L1C4, which suggests that long-time MD simulations are essential to obtaining accurate binding affinities. As shown in Figure 3B, Compound NTP makes hydrogen bonds to antithrombin mainly via its negatively charged sulfate groups. Compound NTP forms hydrogen bonds with Arg13, Arg129, Arg47, and Asn45 with high persistence (70~88%). Compound NTP also forms hydrogen bonds with Arg132, Lys125, and Thr44 with medium persistence (43~66%), and forms relatively weak hydrogen bonds with Arg46, LYS114 and LYS11.

Judging from the hydrogen bond analysis on Compound L1C4 and NTP, Arg47, Arg13, and Asn45 play crucial roles in the antithrombin binding process. Antithrombin provides multiple sulfate/phosphate binding sites consisting of mostly positively charged residues (arginine, lysine) and neutral charged residues that can provide rich hydrogen bond donors/acceptors (asparagine). All four phosphate groups of Compound L1C4 form hydrogen bonds with antithrombin while not all the sulfate groups of Compound NTP can form hydrogen bonds with antithrombin. As pointed out above, introducing positively charged group in the ligand will result in the penalty in solvation free energy. If adding a positively charged group cannot form favorable interactions (e.g. hydrogen bonding), ligand efficiency will be reduced, explaining why that Compound L1C4 has higher ligand efficiency than Compound NTP.

Compare the results from single-structure and ensemble-average MM/GBSA rescoring, the latter yields much accurate results. The ensemble-average MM/GBSA rescoring ranks the binding affinities of antithrombin ligands in the order that agrees with the experimental results. The advantage of ensemble-average MM/GBSA rescoring is that the binding affinity is averaged from ensemble of structures extracted from long-time MD simulations. Long-time MD simulations can explore more configuration space and find energetically favorable configurations, which could offset the bias of initial structures. This can be verified in the MD trajectory of Compound L1C4. Arg13 was observed to form hydrogen bonds with the phosphate group of Compound L1C4 after 8 ns of MD simulation. Our virtual screening pipeline uses a down-select scheme to screening large virtual compound database. A standard procedure to run the pipeline is to down-select compounds after they pass each screening methods implemented in the pipeline. The first screening method in the pipeline is VinaLC docking, which can dock one million compounds in 1.4 h on about 15K CPUs [2]. Top ranked poses of down-selected ligands after docking will be rescored using single-structure MM/GBSA rescoring method. Finally, the most expensive ensemble-average MM/GBSA rescoring method in the pipeline can be applied to the ligands down-selected after single-structure MM/GBSA rescoring.

4 Conclusion

In this article, we introduce a new addition, ensemble-average MM/GBSA rescoring, to our virtual screening pipeline. As a proof of concept, we calculated the binding affinities of seven antithrombin ligands by employing the previous single-structure MM/GBSA rescoring method and newly developed ensemble-average MM/GBSA rescoring method. The correlation coefficient of calculated and experimental binding affinities was improved from 0.36 to 0.69 when uses ensemble-average MM/GBSA rescoring. The rank order of calculated binding free energies using ensemble-average MM/GBSA rescoring exactly matches the experimentally derived free energies. We demonstrate that long-time MD trajectory can explore more configuration space and find energetically favorable configurations so that it can offset the bias of initial structures and improve the accuracy of binding affinity prediction. The electrostatic interactions in both solute and solvent contribute favorably to the binding free energy. Adding more negatively charged groups to the ligand provides more favorable elec-

trostatic energy change. However, it creates a higher penalty for the solvation free energy simultaneously. The penalty can be compensated for by forming more hydrogen bonds as more negatively charged groups are added into the ligand. Compound L1C4 has higher ligand efficiency because it uses all its phosphate groups to form hydrogen bonds with antithrombin while Compound NTP does not.

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